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Original Article

FORMULATION OF NANOSTRUCTURED LIPID CARRIER GEL FROM CALLUS EXTRACT OF MULBERRY LEAF (*MORUS ALBA* L.) WITH 2, 4-DICHLORO PHENOXY ACETIC ACID AND BENZYL AMINO PURINE AS CALLUS GROWTH FACTOR

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ABSTRACT

Objective: This research aimed to formulate the callus extract of mulberry leaf in the form of a Nanostructured lipid Carrier (NLC) gel.

Methods: Dichlorophenoxyacetic acid (2,4–D) and Benzyl Amino Purine (BAP) was used as a callus growth factor. Callus leaf extracted with ethanol 96% by maceration-sonication method. An amount of 0.5% callus leaf extract was formulated into NLC. The NLC is then evaluated for its particle size and polydispersity index. The NLC gel is evaluated for its organoleptic, homogeneity, viscosity, flow ability, and pH. The callus extract and the NLC gel were also evaluated for their tyrosinase inhibitor activity.

Results: The best formulation of NLC showed a particle size of 189.8 nm and a polydispersity index of 0.578. The NLC is a semi-solid, yellowish, odorless, homogeneous gel, with viscosity of 26,666.67 cPs, plastic-thixotropic type, pH of 5.26. The evaluation of tyrosinase inhibitory activity of the callus extract and the NLC gel showed IC_{50} value of 217.64 and 248.12 ug/ml.

Conclusion: It can be concluded that leaf callus extract of mulberry can be formulated into an NLC gel that is physically and chemically stable and has good skin-lightening activity.

Keywords: *Morus alba l.*, Callus leaf, Tyrosinase inhibitor, Skin lightening, Nanostructured lipid carrier

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INTRODUCTION

Every year, nearly 1,500 of new compounds are found that come from plants, 20% of which have certain biological activities [1]. The increasing need for drugs has an impact on the increasing need for producing plants. Lately there are many medicinal plants in the market that are very scarce. Rapid advances in the field of plant cell biotechnology provide new opportunities to produce natural medicinal compounds through tissue culture techniques.

In the pharmaceutical field, tissue culture techniques are very beneficial because they can produce secondary metabolites for the need for large amounts of drugs in a short time [2]. For example, some pharmaceutical industries use tissue culture techniques to produce metabolite products such as alkaloids, in this case codeine from the *Papaver somniferum* plant which is used as an analgesic [3].

Advantages of procuring seedlings through tissue culture it can be obtained superior plant material in large quantities and uniformity, and also sterile culture (mother stock) that can be used as material for further propagation. To get optimum results, the use of basic media and growth regulators is an important factor. The right combination of basic media and growth regulators will increase cell division activity in the process of morphogenesis and organogenesis [4].

Mulberry (*Morus alba* l.), a member of Moraceae family, is a source of stylben's phenol compounds, including resveratrol (3, 5, 4'-trihydroxy-trans-stilbene) and oxyresveratrol (2, 4, 3 ', 5'-tetrahydroxy-trans stilben) are very potential in the cosmetic bioindustry. Oxiresveratrol has been investigated as a tyrosinase inhibitor to avoid excessive production of melanin in the epidermal layer so that it can be used as a skin-lightening agent. It has been reported that oxyresveratrol has a very high IC_{50} value of 1.2 ppm and is used as a depigmentation agent in skin-lightening cosmetic products. Oxyiresveratrol in mulberry extract (*Morus alba* l.) showed very high activity as a tyrosinase inhibitor, with 97% inhibition percentage [5].

In this study, mulberry plant callus culture was made using explants derived from the leaves. As a callus growth media, Murashige and Skoog (MS) media are used in combination with the hormone dichlorophenoxyacetic acid (2,4-D) and benzyl amino purine (BAP) as growth regulators. 2, 4-D is used to stimulate callus induction and BAP is used to stimulate division. 2, 4-D is auxin group and BAP is the cytokinin group. Both have advantages as growth regulators, which are more stable, not easily broken down by enzymatic reactions produced by plants and resistant to heating in the autoclave sterilization process at 121 °C [3, 4]. This is a major factor supporting the success of an *in vitro* culture.

Mulberry leaf callus, which obtained from the tissue culture process, was then extracted and made into Nanostructured lipid Carriers (NLC) with the solvent evaporation method, which is one of the most frequently, used methods for nanoparticles. In this method, the polymer solution is prepared in a volatile solvent and a formulated emulsion. The fat used in making NLC is a combination of oleic acid as liquid fat and cetyl alcohol as solid fat. NLC of mulberry leaf extract obtained was then formulated into a gel preparation that can provide tyrosinase inhibitory activity and meet the quality requirements.

MATERIALS AND METHODS

Materials

Mulberry leaf was obtained from Bogor, West Java. It has been determined as *Morus alba* l. in the Botanical Garden Conservation Center, National Research and Innovation Agency (BRIN), Bogor, West Java, Indonesia, with certificate No. B-1692/IPH.3/KS/V/2018.

Chemical and reagents

Murashige and Skoog solution (MS Media), agar, sugar, and stock of cytokinin growth regulators (BAP) and auxin (2,4-D), 96% ethanol extract from mulberry leaf callus, Kojic Acid (Thornhill, Canada) as standard of tyrosinase inhibitor agent, l-DOPA (SIGMA-Aldrich), tyrosinase from Mushroom (SIGMA-Aldrich), phosphorus buffer solution, potassium dihydrogen phosphate, sodium hydroxide, oleic acid, cetyl alcohol, tween 80, propylene glycol, acetone, carbomer 940, triethanolamine, propylparaben, methyl paraben, and pure water.

Equipments

Culture containers, culture shelves, hand sprayers for alcohols, refrigerators, laminar air flow cabinets, autoclave, alcohol lamps, drop pipettes, aluminum foil, filter paper, tissue paper, dissection equipment (scalpels, tweezers, spatulas, scissors and needles), glassware (pyrex), volumetric equipment (volume pipettes, scale pipettes, water flasks), rotary evaporators, mortars and pestles, water baths, vaporizer cups, analytical balance (AND type GR 200), hot plates magnetic stirrers, SONICA ultrasonic cleaner, ultra turax (IKA Ultra-Turrax, T25, Germany), ELISA Reader (490 nm), Particle Size Analyzer (Malvern, Swiss), Stormer viscometer, stirrer (Eurostar), pH meter (Ultra BASIC) and digital camera.

Preparation of tissue culture media

As much as 34.66g/l of MS media were put into a 1 l beaker glass, then added 30g/l of sugar and 7-8 g/l of agar, then added 2,4-D as much as 1 ppm and BAP as much as 1 ppm, then aquadest was added to the boundary markers, and all of the mixture were stirred until homogeneous. The pH of the final solution is adjusted to around 5.8. The media solution was then heated until it dissolved. Media that is still hot is inserted into sterile culture bottles that have been prepared with a volume of 10 ml each. Then the container was closed with aluminum foil, and sterilized in an autoclave at 121 °C with a pressure of 15 psi for 20-30 min. Sterilized media is stored in the culture media room and ready for use.

Sterilization of mulberry leaves (*Morus alba* l.)

The explants used were mulberry leaves, which had been washed and sterilized gradually, i. e., washed with detergent 1 g/100 ml for 15-30 min, then rinsed with sterile water for 3 min. Next, the leaves are washed with 1 g/100 ml fungicide for 15-30 min and rinsed with sterile water for 3 min. Next, mulberry leaves are washed with 1 g/100 ml of bactericide for 15-30 min and rinsed with sterile water for 3 min. Next, mulberry leaves are soaked with a 5 mg/100 ml streptomycin solution for 1-7 h, and rinsed with sterile water for 3 min. Mulberry leaves are shaken with 10 mg/100 ml $HgCl_2$ solution for 7 min, and rinsed with sterile water for 3 min. Mulberry leaves are shaken with 2% sodium hypochlorite solution for 7 min, and rinsed with sterile water for 3 min. Finally, the mulberry leaves are shaken with sterile water for 3 min for three repetitions.

Planting (initiation) and explant incubation

Sterilized mulberry leaves are planted in MS+ growth promoting substances media; each culture bottle is filled with 1-2 mulberry leaves,±1 cm in size, then covered with plastic and aluminum foil. Each culture bottle containing mulberry leaf explants was then placed on a culture rack which was illuminated with an intensity of 600 lux at 20 °C. The culture then incubated and observed for several months to get a callus ready for harvest.

Extraction of callus leaf

The harvested callus was macerated by the maceration-sonication method using 96% ethanol with a ratio of 5 ml: 1 g. A total of 96.2 g of mulberry leaf callus were macerated with sonerator with 96% ethanol as much as 481 ml for 30 min. Remaceration was carried out three times with 96% ethanol solvent. Furthermore, the liquid extract obtained was evaporated with a rotary evaporator until 11.3 g of thick extract was obtained.

Preparation of NLC of mulberry leaf callus extract

For every 50 ml of the formula NLC mulberry leaf extract, consisting of a 2% mixture of cetyl alcohol and oleic acid (40:60); 4% tween 80; 7% propylene glycol; 0.5% mulberry leaf callus extract; 6 ml acetone; and pure water up to 50 ml. The procedure for making NLC of mulberry leaf callus extract is as follows (24): weigh the ingredients used. Solid fat (cetyl alcohol) and fatty oil (Oleic Acid) (40:60) are mixed and melted in a vaporizer cup at water bath at 85 °C, then added with 96% ethanol extract of callus mulberry leaves that have been dissolved with acetone (Mass 1). Pure water and surfactants (Tween 80 and Propylene glycol) are heated at 85°C (Mass 2). Mass 1 is added to the mass 2 mixture by stirring using a magnetic stirrer at a speed of 600 rpm. The emulsion was stirred with ultraturrax at a speed of 24,000 rpm for 15 min, then the results of the nanoemulsion were cooled with water at a temperature of 2-5 °C by stirring rapidly to produce nanoparticles.

Characterization of nanoparticles

The nanoparticles produced were characterized by particle size and polydispersity index using Particle Size Analyzer (PSA). Zeta potential was measured using Malvern Zetasizer 2000 (Malvern, UK). The NLC suspension was diluted with distilled water (1: 100) to obtain a uniform dispersion and measured at 25 °C. The conductivity of the sample is measured to determine the detection model. The morphology of nanoparticles and the structure of NLC colloids were observed by means of Transmission Electron Microscopy (TEM) [6].

Preparation of NLC gel of mulberry leaf callus extract

Every 100 ml of NLC gel contains 50% w/w NLC of mulberry leaf callus extract, 0.3% w/w Carbomer 940, 0.3% w/w triethanolamine, 5% w/w propylene glycol, 0.01% w/w methylparaben, 0.01% w/w propylparaben, and pure water up to 100 ml. As many as 0.3 g of 940 carbomer; 0.3 g triethanolamine; 50 g NLC mulberry leaf extract; and 5 g propylene glycol are weighed. Carbomer 940 is dispersed into pure water with a volume of 30 times of its weight. Triethanolamine is added gradually while homogenized with stirrer to form a gel base (mixture A). NLC of weighed mulberry leaf callus was added gradually into mixture A until homogeneous. Furthermore, propylene glycol, propylparaben, methylparaben and pure water were added, and then homogenized with a stirrer at 500 rpm for 15 min [7].

Physical and chemical evaluation of gel preparations

Evaluation of gel preparations includes physical evaluation (organoleptic, viscosity, flow properties, and homogeneity, and chemistry (pH). Viscosity and flow properties are carried out using a Stormer viscometer. Homogeneity was observed by applying the gel preparations on the slide and coated with another slide, after which it was observed whether the gel was homogeneous or not. Test of the enzyme inhibitory activity of tyrosinase extract and NLC gel of mulberry leaf callus *in vitro* [7].

Preparation of kojic acid standard curve

As a substrate, a 10 mmol l-DOPA solution is used in a 50 mmol phosphate buffer pH 6.8. The tyrosinase enzyme used was a concentration of 75 U/ml in a phosphate buffer of 50 mmol pH 6.8. Prepared kojic acid solution with a concentration of kojic acid solution 50, 20, 10, 5, and 2.5 µg/ml in phosphate buffer 50 mmol pH 6.8 to create a standard curve. Each positive/standard control reaction mixture consisted of 70 µl kojic acid solution, 110 µl l-DOPA, and 30 µl tyrosinase enzyme solution, then put into 96 well-microtiter plates. For each concentration, a blank is made in which no tyrosinase enzyme solution is added. The plates were then incubated for 20 min at 37 °C. The mixture is measured in absorbance using a microplate reader at the optimum wavelength.

Inhibitory activity test of tyrosinase enzyme from mulberry leaf (*Morus alba* l.) extract

A total of 2 ml of mulberry leaf callus extract was dissolved in 10.0 ml of 96% ethanol to obtain a concentration of 1,000µg/ml. The solution was pipetted 1 ml and put into a 10.0 ml volumetric flask. The volume of the flask is sufficient with a phosphate buffer pH of 6.8 to obtain a solution concentration of 100µg/ml and so to obtain a concentration of 200, 300, 400 and 500 µg/ml of the solution. Then, a total of 70µl extract solution mixed with 110 µl l-DOPA solution and 30 µl tyrosinase enzyme solution then put into 96-well microtiter plates. Each sample was made blank, where tyrosinase enzyme solution was not added. The plates were incubated during the optimum incubation time of 20 min at 37 °C. The mixture is measured in absorbance using a microplate reader at the optimum wavelength.

Inhibition of the enzyme activity of tyrosinase gel NLC gel inhibition of mulberry leaf (*Morus alba* l.) extract *in vitro*

A total of 4 ml of NLC gel of mulberry leaf callus extract was dissolved in 10.0 ml 96% ethanol to obtain a concentration of

1,000µg/ml. As much as 1 ml of the solution was pipetted and poured into a 10.0 ml volumetric flask. The volume of the volumetric flask is sufficient with a phosphate buffer pH of 6.8 to obtain an NLC solution concentration of 100 µg/ml and thus to obtain a concentration of NLC solutions of 200, 300, 400 and 500 µg/ml. At the next step, a total of 70 µl of NLC gel solution, 110 µl of l-DOPA solution, and 30 µl of tyrosinase enzyme solution then put into 96-well microtiter plates. Each sample was made blank where tyrosinase enzyme solution was not added. The plates were incubated during the optimum incubation time of 30 min at 37 °C. The absorbance of the mixture is measured using a microplate reader at the optimum wavelength.

Calculation of the tyrosinase and IC_{50} enzyme inhibitory activity

The measured absorbance is the absorbance of dopachrome formation. From the absorbance of this measurement, the percentage of tyrosinase inhibition can be calculated according to the method with the following formula:

$$\% \text{ tyrosinase inhibition} = \text{ x } 100\%$$

B= absorbance minus control/blank (B)

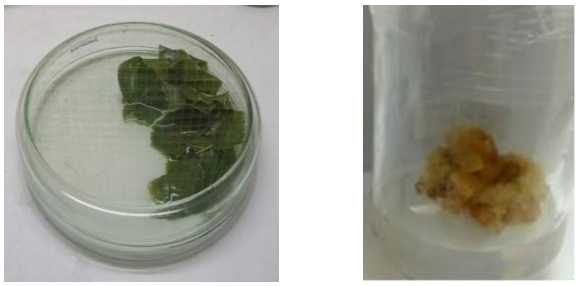
S = absorbance of the sample minus the sample blank (S)

IC_{50} was calculated using the absorbance of the linear regression equation, sample concentration (in logarithms) as the x-axis and percent inhibition (% inhibition) as the y-axis. From the equation y = a+bx, the IC_{50} values can be calculated.

RESULTS

Mulberry leaves callus growth

Mulberry leaf explants and callus are presented in fig. 1.



(a) **(b)**

Fig. 1: Explants (a) and callus (b) of mulberry leaves (*Morus alba* l.)

The formation of callus also comes from the supply of natural or artificial hormones from the outside in explants. Callus growth percentage of mulberry leaves is presented in fig. 2.

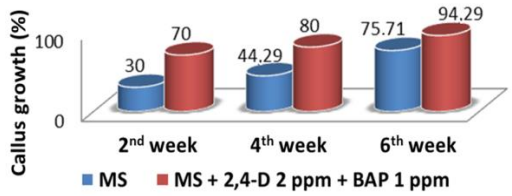


Fig. 2: Growth of mulberry leaves callus on initiation media (MS_0) and MS media with additional growth factor (2,4-D 2 ppm+BAP 1 ppm)

Production of NLC from mulberry callus leaves

Solvent evaporation is one of the most frequently used methods for nanoparticle preparation. In this method, the polymer solution is prepared in a volatile solvent and a formulated emulsion. The results of examinations on various formulas produce a mixture of fat with a concentration of 2% with all variations of surfactant concentrations, resulting in a liquid NLC form, in the form of white nanoparticles, a little foam, stable at room temperature and no deposits.

Characterization of NLC nanoparticle

The size of the NLC particles was measured by particle size analyzer. Measurements were taken at 25 °C [9]. From the measurement results, obtained NLC particle size of 189.8 nm with a polydispersity index of 0.578. The potential zeta on NLC of mulberry leaf callus extract was-7.37 mV.

Observation of morphology of NLC gel of mulberry leaf callus using TEM JEOL 1010, 80.0KV with a magnification of 25,000 times can be seen in fig. 3. From the picture, visible round-shaped particles surrounded by the outer sides of lipid particles and the inner side in the form of leaf callus extract particles mulberry. In the observation of TEM analysis, results obtained a particle size of 200 nm.

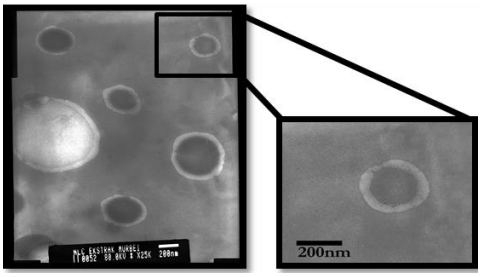


Fig. 3: TEM results of NLC of mulberry leaf callus extract

Evaluation of NLC gel callus extract of mulberry leaves

Evaluation of NLC gel of mulberry leaf (*Morus alba* l.) extract includes organoleptic test, homogeneity, viscosity and flow properties, and pH of NLC gel callus from mulberry leaf extract. Following are the evaluation data of NLC gel mulberry leaf extract.

Organoleptic evaluation of NLC gel of mulberry leaf callus showed appearance with green color, had a semi-solid consistency with a slightly viscous shape, and was odorless, whereas on clear blanks, it had a semi-solid consistency with a thick and odorless form. Based on the results of homogeneity evaluation tests on blank formulas and NLC gel, mulberry leaf extract obtained homogeneous preparations.

Evaluation of the viscosity and flow properties of NLC gel preparations was performed using a Brookfield lV type viscometer. Based on the test results, the viscosity of the blank was 34,833.33 cPs and the viscosity of the gel was 26,666.67 cPs. The results of the evaluation of the flow properties of NLC gel of mulberry leaf callus extract are presented in fig. 4. From fig. 4, it can be seen that the flow properties of NLC gel callus and mulberry extract are plastic thixotropic. The pH of the blank preparations is smaller when compared to NLC gel preparations, which are 6.20 and 5.26, respectively.

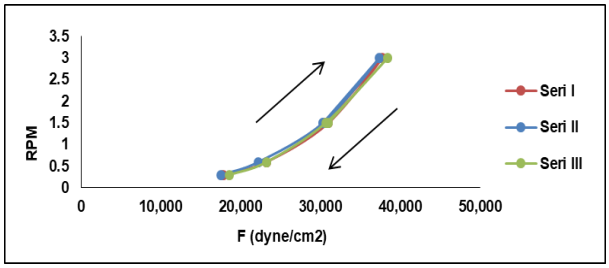


Fig. 4: Flow evaluation of NLC gel formula of mulberry leaf callus extract

Inhibitory activity of tyrosinase extract and NLC gel callus extract of mulberry leaf *in vitro*

The results of the enzyme inhibition activity of mulberry leaf *(Morus alba* l.) callus extract showed an average IC_{50} value of 217.64 µg/ml. This value indicates the inhibitory activity of the weak tyrosinase enzyme. Meanwhile, the results of measurement of the tyrosinase inhibitory activity of NLC gel had an average IC_{50} of 248.12µg/ml.

DISCUSSION

Mulberry leaves callus growth

One of the factors that support the success in growing callus culture is growth media and growth regulators. In this study, Murashige and Skoog media (MS Media) and growth regulators dichlorophenoxyacetic Acid (2,4-D) with a concentration of 2 ppm and Benzyl Amino Purin (BAP) with a concentration of 1 ppm were used. MS media was media with high ion concentrations compared to Woody Plant Medium (WPM) media, which have lower ions which are suitable for woody explants. Dichlorophenoxyacetic acid (2, 4-D) is an auxin group and Benzyl Amino Purin (BAP) is a cytokinin group. Both of these growth regulators have their own advantages, which are more stable because they are not easily broken down by the reaction of enzymes produced by the plant and are resistant to heating in the autoclave sterilization process at 121 °C [10].

From the observation it was found that the emergence of the callus came from the injured part or explant incision in contact with growing media. Callus growth begins with swelling in the explant and the incision will swell (swelling), this occurs because of the stimulation of the tissue and the response to growth regulators in the wound part of the plant [11].

Based on fig. 2, it can be seen that the MS media with the addition of growth promotion substances (2,4-D 2 ppm and BAP 1 ppm) have the largest percentage of the number of callus growth compared to MS media without the addition of growth promotion substances. The results of this study showed the percentage of callus induced in the treatment of 2,4-D 2 ppm and BAP 1 ppm was able to induce the fastest callus on day 14 by 70% in all callus culture media samples. While the percentage of callus induced in the MS_0 media treatment was able to induce late callus on the 14th day by only 30%. A very small percentage of callus growth in MS_0 media can be caused by the absence of hormones that can stimulate quickly and cannot increase the process of callus cell division. A large percentage of callus growth in MS media with the addition of 2,4-D 2 ppm and BAP 1 ppm can be caused because the 2,4-D hormone is most often used in inducing callus because it has a strong activity to stimulate the process of cell differentiation, suppress organogenesis and maintain plant callus growth. The 2,4-D growth regulator used has a carboxyl group separated by carbon or carbon and oxygen, which optimizes the 2,4-D activity in callus formation, while the BAP hormone stimulates cell division, morphogenesis and callus proliferation. BAP added to culture media will increase the rate of protein synthesis so that it encourages rapid cell enlargement and division. The combination of 2,4-D and BAP in MS media must be optimal and equal, so that it can induce callus growth. Based on observations of mulberry leaf callus has a compact callus texture so that it can accumulate a lot of secondary metabolites in it and callus color results obtained by MS media with the addition of 2,4-D 2 ppm and BAP 1 ppm are green in color, which indicates there is a lot of chlorophyll in it at the beginning of callus growth, but over time, it turns brownish white due to an increase in phenol levels in the callus [12].

Production of NLC from mulberry callus leaves

Solvent evaporation is one of the most frequently used methods for nanoparticle preparation. In this method, the polymer solution is prepared in a volatile solvent and a formulated emulsion [13]. This method utilizes high-speed homogenization or ultrasonication, followed by solvent evaporation, either by continuous magnetic stirring at room temperature or at low pressure [8]. From the optimization results, the best amount of acetone to dissolve the extract without producing residual acetone is 6 ml. The optimal ratio of oleic acid and cetyl alcohol in the formula is 40:60. In this formula a mixture of homogeneous, solid-shaped materials which have a consistency that is not too hard and not too soft and has good stability when stored at room temperature. Based on organoleptic examination results, the higher levels of oleic acid the more dilute the lipid mix consistency. The formulated NLC showed physically stable results because the NLC color remained white, there was no foam and no deposits were found. The results of examinations on various formulas produce a mixture of fat with a concentration of 2% with all variations of surfactant concentrations resulting in a liquid NLC form, in the form of white nanoparticles, a little foam, stable at room temperature and no deposits.

Characterization of NLC nanoparticle

Characterization is carried out generally as initial characterization of particle size, polydispersity index, and in the advanced characterization of potential zeta values, and morphological observations using Transmission Electron Microscope (TEM) [14]. The size of the NLC particles that have been made are measured by a Malvern device. Particle size analysis was performed using Dynamic light Scattering (DLS) technique [15]. DLS is used to measure particle size in the submicron range using Brownian motion in aqueous media. Measurements were taken at 25 °C [9]. From the measurement results, obtained NLC particle size of 189.8 nm with a polydispersity index of 0.578. The size of nanoparticles used in cosmetics is 10-100 nm but the size of 200-300 nm is still acceptable because the pore size of the skin is much larger scale in micrometers, so the NLC formula of mulberry leaf callus extract can be used for gel preparations. Polydispersity index results which show the size distribution of nanoparticles. The condition of the polydispersity index value is 0-1, where the polydispersity index value approaching 0 indicates a uniform nanoparticle size distribution while the polydispersity index value of more than 1 indicates that the sample has a very wide size distribution and can contain large particle sizes or aggregates which can slowly experiencing sedimentation.

Potential Zeta usually ranges from+100 to-100 mV [16]. The magnitude of the zeta potential can predict colloidal stability. Nanoparticles with zeta potential values greater than+25 mV or less than-25 mV usually have a high degree of stability. Dispersion with low zeta potential value will produce aggregate. The potential yield of zeta on NLC of mulberry leaf callus extract was-7.37 mV. The potential zeta value obtained fulfills the requirements, which is between+25 mV to-25 mV.

Observation of morphology of NLC gel of mulberry leaf callus using TEM JEOL 1010, 80.0KV with a magnification of 25,000 times can be seen in fig. 3. From the picture, visible round-shaped particles surrounded by the outer sides of lipid particles and the inner side in the form of leaf callus extract particles mulberry. In the observation of TEM analysis results obtained a particle size of 200 nm.

Evaluation of NLC gel callus extract of mulberry leaves

Evaluation of NLC gel of mulberry leaf (*Morus alba* l.) extract includes organoleptic test, homogeneity, viscosity and flow properties, and pH of NLC gel callus from mulberry leaf extract. Following are the evaluation data of NLC gel mulberry leaf extract.

Organoleptic evaluation of NLC gel of mulberry leaf callus showed appearance with green color, had a semi-solid consistency with a slightly viscous shape, and was odorless, whereas on clear blanks, it had a semi-solid consistency with a thick and odorless form.

Homogeneity evaluation aims to determine whether the entire mixture of ingredients from the gel preparations made have been mixed properly or not, the test is done by looking visually (five senses) in the hand area or looking at the surface of the glass object. Based on the results of homogeneity evaluation tests on blank formulas and NLC gel, mulberry leaf extract obtained homogeneous preparations. This is obtained from the process of making gel preparations with stirring at a certain speed and optimal stirring time. Homogeneous gel preparations are expected to contain active substances that are spread evenly in the preparations so as to be able to give the desired and uniform effect on each use as skin lightening cosmetics [17].

Evaluation of the viscosity and flow properties of NLC gel preparations was performed using a Brookfield lV type viscometer. Based on the test results, the viscosity of the blank was 34,833.33 cPs and the viscosity of the gel was 26,666.67 cPs. The more NLC of Mulberry callus extract is added to the formula, the lower the viscosity and resistance. Viscosity states the resistance of a liquid to flow. The higher the viscosity, the higher the resistance. Flow properties are obtained by making a graph between the force (dyne/cm) and rpm in accordance with the data obtained; then, the data is plotted between forces (x) and rpm (y), then the flow properties are determined. The results of the evaluation of the flow properties of NLC gel of mulberry leaf callus extract are presented in fig. 4. From fig. 4, it can be seen that the flow properties of NLC gel callus and mulberry extract are plastic thixotropic.

This plastic flow characteristic is the expected flow type in the preparation because when it is applied, it will flow in the presence of pressure (shearing stress) that passes through the yield value. This also ensures that the NLC gel will be stable in storage and comfortable to use.

Evaluation of pH aims to analyze whether the pH of the preparation is suitable with the pH of skin, so as not to cause irritation. Based on the evaluation of pH, blanks and NLC gel, mulberry leaf extract has different pH values. The pH of the blank preparations is smaller when compared to NLC gel preparations, which are 6.20 and 5.26, respectively. This increasing of pH has a good effect on the stability of the preparation because the gelling agent used (carbomer 940) is stable at pH 5-7, and the oxyresveratrol compound is also stable on the gel preparation because the pH of oxyresveratrol is 5.38. The pH of the preparation obtained still meets the requirement of pH scale that is allowed in facial skin preparation, which is 4.5-6.5, so that if used, it will not cause irritation on the skin.

Inhibitory activity of tyrosinase extract and NLC gel callus extract of mulberry leaf *in vitro*

Evaluation of tyrosinase inhibitory activity was carried out on mulberry leaf callus extract. Kojic acid was used as a positive control, which could inhibit enzymatic oxidation of tyrosine in a non-competitive manner and using l-DOPA as the substrate [18]. The tyrosinase enzyme will produce dopachrome compounds which will then form a melanin. If the mulberry leaf callus extract has a tyrosinase inhibitor, the activity of the tyrosinase enzyme will be inhibited in producing the melanin. *In vitro* tyrosinase inhibition test was determined by measuring the absorbance using a microplate reader at the optimum wavelength with ELISA Reader and IC_{50} values were obtained as parameters of the tyrosinase enzyme inhibition ability.

The principle of this test is the measurement of dopachrome products from the l-DOPA oxidation reaction with the enzyme tyrosinase, which is characterized by the formation of a brownish orange color [19]. The presence of inhibitors causes the reaction of l-DOPA with the tyrosinase enzyme running slowly which is characterized by a decrease in the intensity of the dopachrome color. Determination of the color intensity of dopachrome is measured using a microplate reader. The absorbance obtained is used to find out how much inhibitor activity in inhibiting the reaction of l-DOPA with the tyrosinase enzyme. The incubation time to see enzyme inhibitory activity is 20 min at 37 °C [11]. Absorbance measurements were carried out at wavelength λ 490 nm, enzyme concentration of 75 U/ml, and substrate concentration of 10 mmol [7].

The results of the enzyme inhibition activity of mulberry leaf *(Morus alba* l.) callus extract showed an average IC_{50} value of 217.64 µg/ml. This value indicates the inhibitory activity of the weak tyrosinase enzyme. Meanwhile, the results of measurement of the tyrosinase inhibitory activity of NLC gel had an average IC_{50} of 248.12µg/ml. This value showed a decrease in inhibitory activity when compared to pure mulberry leaf callus extracts. This can be caused by the percentage of the extract content of NLC formulated into a gel preparation reduced levels that previously contained 0.25 g/50 ml NLC to 0.25g/100 ml gel, and can be caused by the influence of the formulation on the activity of the enzyme tyrosinase gel. The weak tyrosinase inhibitory activity value can be caused by the callus used is still 2-3 mo old and not optimal enough in producing secondary metabolites. The longer the time of the callus life and the larger biomass of the callus, as equal, the higher the level of secondary metabolites it could generate [20].

CONCLUSION

Dichlorophenoxyacetic Acid (2,4-D) and Benzyl Amino Purin (BAP) as growth regulators can induce mulberry (*Morus alba* l.) leaf callus growth in the second week by 70% induced, 4th w by 80% induced and the 6th w by 94.29%. Mulberry leaf callus extract can be made into NLC gel preparations with organoleptic characters as follow: green, semi-solid, odorless, homogeneous, viscosity of 26,666.67 cPs, plastic thixotropic flow properties, pH of 5.26, particle size of 189.8 nm, and polydispersity index of 0.578. Both of the callus extracts and the NLC gel of callus extracts have weak tyrosinase enzyme inhibitory activity with IC_{50} values of 217.64 µg/ml and 248.12 µg/ml, respectively.

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AUTHORS CONTRIBUTIONS

Faizatun: conceived and planned the experiments, contributed to the interpretation of the results of NLC, and led in writing the manuscript; Nur Miftahurrahmah: conceived and planned the experiments, and contributed to the interpretation of the results callus preparation

Abul 'ala Maududi: carried out the experiments and contributed to sample preparation of callus and nanoparticle lipid carriers; Diah Kartika Pratami: contributed to the interpretation of the results of the activity tyrosinase test of NLC and revised the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

CONFLICT OF INTERESTS

The Authors declare no conflicts of interest.

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